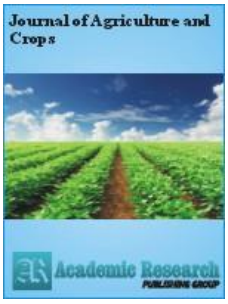

Journal of Agriculture and Crops
ISSN: 2412-6381
Vol. 1, No. 2, pp: 26-32, 2015
URL: http://arpgweb.com/?ic=journal&journal=14&info=aims

Predicting of the Embryogenic Performances of 5 Upper Amazon Cocoa Parents Using the Discriminant Model of Wilks' Lambda

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Abstract: To predict the embryogenic performances of 5 upper amazon cocoa parents, the discriminant model of Wilks' Lambda was used. Five parents, namely IMC67, P19A, Pa13, Pa121 and Pa150 were used. Staminodes and petals from these parents were extracted of flower buds then cultured *in vitro* onto 2 callogenesis media, namely PCG1 and PCG3. The Principal Component (PCA), Hierarchical Cluster HCA) and Factorial Discriminant Analyses (FDA) were performed. As for the PCA, a single, namely the number of callogenic explants, out of 5 measured variables, was dropped from the study. Two classes were identified from the PCA and HCA, then confirmed via the FDA. Clones P19A and Pa13 belonging to Class C2, displayed the highest embryogenic performances. Earlier study relying on multivariate analysis implying hybrids and these 5 cocoa parents revealed results approximately similar. The model predicting the embryogenic performances is spelt Z1 = -3.310 + 4.532*Ncalem. In oil palm, 2 models, the first one predicting the biomass production and the second one modeling the number of harvested shoots from somatic embryos were proposed. From model proposed here, the embryogenic performances of the best genotypes will vary from 1.3725 to 1.7402. Their discriminating score Z1 will oscillate from 2.910 to 4.576. This model will allow the predicting of membership class of a new observation from its values of the number of embryogenic explants.

Keywords: Membership class; PCG media; SCG medium; ED medium; Predicting equation; Communality.

1. Introduction

Cocoa tree is a cross-pollinated forest species originated from the rainforest of the South and Central America [1]. It belongs to the Malvaceae [2]. *Theobroma cacao* L., is the only cultivated species from genus Theobroma used for manufacturing of chocolate. Côte d'Ivoire is the largest producer of beans from cocoa tree. Its yielding accounts for about 44.25% of the global one [3]. More than six millions people depend directly or indirectly on cocoa cultivation. It accounts for 30% of working population [4].

Predictive model of the *in vitro* embryogenic performances of upper amazon cocoa parents has stayed little known. Indeed, the sustainability of cocoa crop in producer countries depends largely on the cloning technique control. In oil palm, Konan, *et al.* [5] suggested the modeling of interactions occurring during rooting of the somatic-embryos derived plantlets. Likewise, in Konan, *et al.* [6], the modeling of the prediction of the amount of culture biomass to be transferred and that of the number of harvested shoots at the end of each culture cycle were carried out. Nonetheless, somatic embryogenesis technique is one of the means used for cocoa cloning [7-9]. The reliability of this technique was tested successfully [10, 11]. With respect to prediction, the 5 upper amazon cocoa parents were already structured, nonetheless in combination with their 6 hybrids [12]. Thus, no study has reported only the *in vitro* embryogenic performances prediction of 5 upper amazon cocoa parents analysed here. The knowledge of this prediction model would allow the rapid vegetative propagation of the best cocoa parents for the setting up of two-clone seed orchard field. Likewise, this knowledge would allow the prediction of membership cluster of a new observation from its somatic embryogenesis values. Moreover, it seems that one variable describing the somatic embryogenesis might predict accurately the embryogenic performances among the 4 used in the study in cocoa parents.

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The objective of work was to search for and identify a prediction model of the *in vitro* embryogenic performances of 5 upper amazon cocoa parents.

2. Materials and Methods

2.1. Plant Material, Experimental Conditions and Design

Plant material was obtained from ancient research station of CNRA (Centre National de Recherche Agronomique) based on Bingerville. The latter is located at 3° 52' 59" West longitude and 5° 21' 42" North latitude and 59 m above sea. From 2002 to 2004, at the meteorological Bingerville Station of CNRA, the weekly pluviometric total, weekly average maximum temperature, weekly average minimum temperature, average relative humidity were 4186.80 mm, 30.63°C, 20.16°C, 77651.42 hours, 82.13%, respectively. Experiment was conducted from January to February 2005. Flower buds were harvested at Bingerville then carried at the Central Biotechnology Laboratory. They were prepared for culture purposes according to Li, *et al.* [7] and Young, *et al.* [13] protocols.

The 5 cocoa parents, namely IMC67, P19A, Pa13, Pa121 and Pa150, were planted in row in the fields B1A, B1B and B10 of the Bingerville site according to a design without randomisation. These rows consisted of clones of each of accessions represented in genebank. Only one cocoa tree for each parental clone was used during the experiment.

With respect to culture initiation, a 5 x 2 x 2 factorial scheme in a modified completely randomised design was used. Modifications concerned the culture of staminodes and petals in the same Petri dish. Treatments were prepared in triplicate. In all, 6 treatments were obtained per genotype. Therefore, the 5 cocoa parents providing each 2 explants (staminodes and petals) were cultured onto 2 culture media. The latter consisted of Li, *et al.* [7]'s media. These are PCG1 and PCG3 for culture initiation, SCG1 for secondary callus growth and ED with respect to embryos development.

2.2. Explants Preparation, Culture Conditions and Media

Flower buds measuring 4-5 mm long were collected on the 5 cocoa parents twice a week, early in the morning. They were used as a source of explants. Primary somatic embryos were obtained as described in Li, *et al.* [7] by culturing staminode and petal explants onto 2 primary callogenesis media namely PCG1 and PCG3. Fourteen days after the culture onto Primary Callus Growth (PCG) media, the callogenic explants were subcultured onto Secondary Callus Growth (SCG). Fourteen days later, callogenic explants were again subcultured onto hormone free Embryos Development medium (ED). Onto this one, they were subcultured threefold every 21 days.

2.3. Variables Measurement

At the end of 3 months of culture, the number of callogenic explants (Ncal), the number of embryogenic explants (Ncalem), the embryos number yielded per embryogenic explant (Nemb) were scored. From these, the average embryos number provided per embryogenic explant (Mece) as well as embryogenesis percentage (Pe) were calculated.

2.4. Statistical Analysis

Collected data were subjected to the Principal Component (PCA), Hierarchical Cluster (HCA) and Factorial Discriminant Analyses (FDA) by means of the softwares SPSS and Xlstat, versions 22.0 and 2007, respectively.

3. Results

3.1. Data Reduction by Means of the PCA

This reduction of collected data was achieved through : i) the analysis of the data factorizability, ii) choice of principal components, iii) the choice of variables and iv) interpretation of the variability observed. Regarding the factorizability, Kaiser-Meyer-Olkin (KMO) coefficient was 0.546, thus greater than 0.5. The Bartlett's test of sphericity was very highly significant (p-value = 0.002). Such a value for p-value is greater than the 0.10 level. The meeting of these 2 conditions allowed the statement that data were factorizable.

As for the choice of used variables, only the number of callogenic explants was dropped. The 4 others were selected for the rest of the study.

Concerning the choice of principal components, the first 2 were selected. They explained 99.86% total variability. As far as the interpretation of variability is concerned, the first component explained 53.23% total variability, as against 46.53% for the second one. It described parents expressing correct value of the mean of the somatic embryos yielding. The second principal component represented parents displaying correct somatic embryogenesis percentage.

3.1.1. Footnote related to the PCA

The analysis of the output from the PCA was carried out through the data factorizability, the choice of variables and that of principal components as well as the interpreting of the variability expressed. Data factorizability was appreciated using the Kaiser-Meyer-Olkin (KMO) coefficient and Bartlett's test of sphericity. Regarding the first one, the KMO was 0.546. Such a value was between 0.5 and 0.6 according to Kaiser's scale. Thus, it was considered to be poor. Nonetheless, it conferred correct factorial structure to data. For Bartlett's test of sphericity, the statistics

of approximate Chi-square, which is associated with it, was 20.946 (p-value = 0.002). This statistics was high. The probability which is associated with it was very smaller than 0.10 postulating that at least one of correlations between variables is significantly other than zero. The meeting of these 2 conditions allowed the continuation of the analysis.

For the choice of variables to use to interpret the variability, the number of explants callogenic was dropped. Indeed, it discarded value of the correlation matrix, and thus made impossible not only the calculation of KMO coefficient, but also the performing of Bartlett's test of sphericity. Likewise, all of 4 selected variables showed correct communalities revealed by their positioning on circle of correlations (Figure 1).

From 4 variables selected like relevant, 4 principal components were extracted using the Kaiser's normalization method.

As far as the choice of principal components is concerned, 2 were selected, because they accounted for more than 70% total variability. The first one, namely component 1, described 53.23% total variability. It is defined by the embryos number per embryogenic explant and the average number of embryos per embryogenic explant. It displayed upper amazon cocoa parents expressing high yielding of embryos (Table 1). The second one, known as component 2, explained 46.63% unexplained variability by component 1. It characterised the proportion of upper amazon cocoa parents yielding the embryogenic explants (Table 1).

The projection of observations on the plane 1-2 of the PCA seemed to reveal the existence of 2 classes. First, would consist of parents IMC67, Pa150 and Pa121, would differ from 2 others by a low mean of embryos yielding. Second, composed of PA13 and P19A, would be distinguishable from the first by a high potential of yielding of embryogenic explants (Figure 1; Table 1).

Figure-1. Communality of 3 measured variables and scatter plot structuring the 5 parents on the plane 1-2 of the correlation circle and that of the factorial map, respectively, from the PCA.

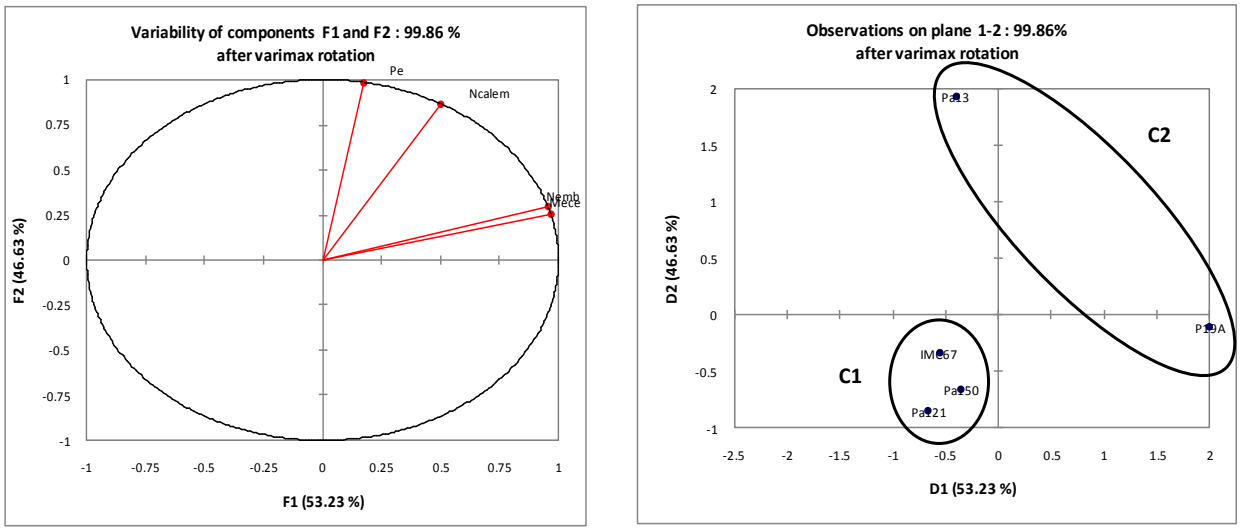


Table 1 : Expressed variability and cosine squared of each of the variables measured on each of principal components 1 and 2.

		F1	F2
	Variability expressed (%)	53.23	46.63
	Cumulative %	53.23	99.86
Cos^2	Ncalem	0.253	**0.744**
	Nemb	**0.913**	0.085
	Mece	**0.933**	0.066
	Pe	0.030	**0.970**

Legend
Cos^2: cosine squared.

3.2. Variability of 5 Upper Amazon Cocoa Parents On Basis of Their Callogenic and Embryogenic Potential from the HCA

As far as the multivariate classification is concerned, the number of observations was smaller than the 100 level. This allowed the choice the HCA instead the k-means multivariate method (http://www.lemoal.org/spss/). The observations were clustered into 2 classes, confirming the reports done in the course of the analysis of the PCA. Each accounted for 60% and 40% of the total number, respectively. Such percentages, very higher than 10% level, allowed the validation of the analysis performed. Furthermore, at level 15 of the scale of dendrogramme the truncation was done. This revealed the existence of differences. The analysis of data displayed that these differences derived from only one variable out of 5 used. Thus, 4 did not discriminate classes, whereas 1 discriminated them (Figure 2; Table 2).

Class C1 consisted of 3 observations, that is to say IMC67, Pa121 and Pa150. It differed from C2 by low number of embryogenic explants (Ncalem), low embryos number per embryogenic explant (Nemb), low average embryos number per embryogenic explant (Mece) and low embryogenesis percentage (Pe). On the contrary, it recorded high number of callogenic explants (Ncal; Figure 2; Table 2).

Class C2 comprised 2 observations, namely P19A and Pa13. It stood out from C1 by high number of embryogenic explants (Ncalem), high embryos number per embryogenic explant (Nemb), high average embryos number per embryogenic explant (Mece) and high embryogenesis percentage (Pe). However, it showed low number of callogenic explants (Ncal; Figure 2; Table 2).

Figure-2. Clustering of the 5 upper amazon cocoa parents from their callogenic and embryogenic values.

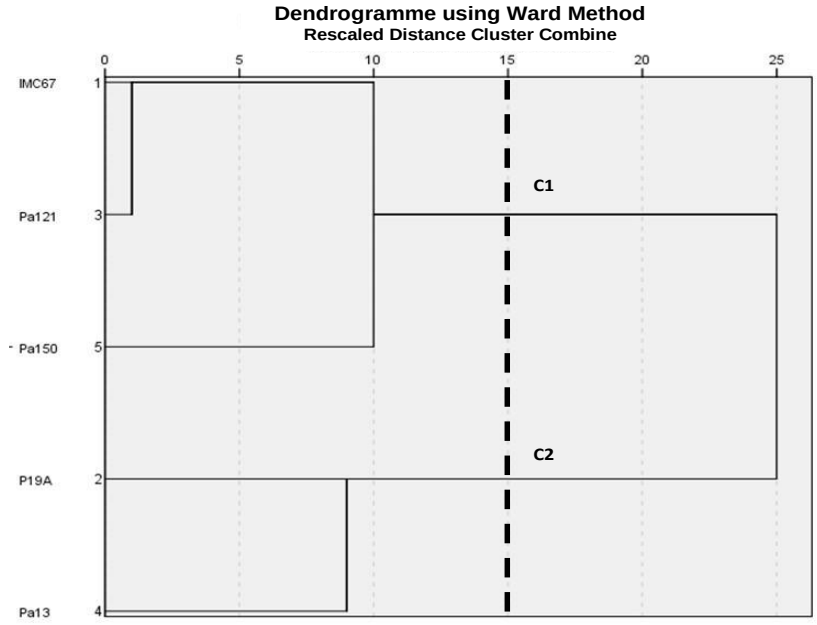


Table-2. Separation of the class means from the HCA based on Student t test.

Class	Ncal	Ncalem*	Nemb	Mece	Pe
C1	15.070a	0.180a	0.474a	0.441a	1.039a
C2	13.686a	1.556b	6.140a	3.629a	8.068a
Mean	14.378	0.868	3.307	2.035	4.553
p-value	0.648	0.006	0.075	0.094	0.069

Legend : Ncalem*: variable expressing the number of embryogenic explants. Different letters accompanying average values in column express significant difference between the 2 means according to Student t test at 5% threshold.

3.3. Predicting of the Somatic Embryos Yielding Based On the Analysis of the Classes Conformity Provided By the HCA

Regarding the FDA, the conformity between the 2 classes was checked using the: i) looking for differences between classes, ii) validation of the Wilks' Lambda method, iii) estimate of the coefficients of the discriminant function and iv) analysis of the communality. Before the assessing of 4 previous points, the relevance of measured variables was analysed. Thus, the number of callogenic explants and the number of embryogenic explants were identified to be relevant. Indeed, they recorded Variance Inflation Factor (VIF) lower than 10 level (VIF / Ncal = 1.513 ; VIF / Ncalem= 3.655). In contrast, the embryos number per embryogenic explant (Nemb), the average embryos number per embryogenic explant (Mece) as well as the embryogenesis percentage (Pe) expressed VIF higher than 10 level (VIF / Nemb = 487.504; VIF / Mece = 455.402; VIF / Pe = ∞). Therefore, the first 2 variables were selected for the rest of the study, while the last 3 were dropped from the same study (Table 3).

The looking for the differences between the 2 classes identified was performed through the examination of variance of measured variables on the one hand, as well as the analysis of Fisher-Snedecor F statistics and Wilks' Lambda on the other hand. For the variance, those from the 2 selected variables appeared to be discriminant (Ncal = 13.419; Ncalem = 0.039). As for the Fisher-Snedecor's F statistics, it was high (Ncal = 0.256; Ncalem = 46.704). Concerning the Wilks' Lambda, that of the 2 variables revealed relevant was lower or equal to 0.90 (Ncal = 0.900; Ncalem = 0.060). Consequently, the analysis of the 3 abovementioned criteria showed the existence of differences between 2 classes identified.

The validation of the Wilks' Lambda method was assessed per step examining : i) the Box's M statistics, ii) the global correlation and iii) the Wilks' Lambda which is associated them. Roughly, stepwise statistics showed that it was possible to extract from 2 initially variables found relevant, only one containing the sufficient information allowing the complete discriminating of the 2 previously identified classes. It concerned the number of embryogenic explants. Indeed, in step 1, the entry of the number of callogenic explants trigerred discrimination of the clusters (λ =

0.060; p-value = 0.006). More specifically, for the Box's M statistics, the variance-covariance matrixes were statistically equal (Box's M = 0.103; p-value = 0.789) postulating that the linear FDA was preferable to the quadratic one. The global correlations tended towards 1, namely 0.969 for the only discriminant function 1. This only discriminant function 1 discriminated the 2 classes in the proportion of 100%. Regarding the Wilks' Lambda linked with the significance of test of the function 1, it was equal to 0.060 with p-value corresponding to 0.080. In short, the Wilks' statistics only allowed the validation of the function 1.

Classes C1 and C2 were placed on the function 1 (Figure 3). C1 was associated with the count of the embryogenic parents, whereas C2 was related to their proportion (Table 2).

From these 2 classes, only one predicting equation was extracted. It is about : Z1 = -3.310 + 4.532*Ncalem.

The communality was assessed using the confusion matrix. It showed that in class C1, 100% observations represented by parents IMC67, Pa121 and Pa150 were well-classified thanks to the predicting equation 1. Likewise, in class C2, 100% observations represented by P19A and Pa13 were correctly classified (Table 4).

With respect to the pairwise distance, the one calculated between C1 and C2 was 42.957. The Hotelling T^2 test associated with this distance was very highly significant (Hotelling T^2 = 107.599; p-value = 0.000; Tables 5 and 6).

Table-3. Searching for relevant variables through the Variance Inflation Factor (VIF).

Statistics	Ncal	Ncalem	Nemb	Mece	Pe
Tolerance	0.661	0.274	0.002	0.002	0.000
VIF	1.513	3.655	487.504	455.402	∞

Legend : VIF* : Variance Inflation Factor calculated from formula 1 / Tolerance. The latter itself is calculated from formula 1 – R^2, where R^2 represents the coefficient of determination expressing the fit degree of the data to model. It is calculated as follows : R^2 = 1 – sum of squares (factorial) / sum of squares (residual).

Table-4. Examination of the communality based on the confusion matrix

			Membership class predicted		
		Ward method	C1	C2	Total
Original	**Count**	C1	3	0	3
		C2	0	2	2
	%	C1	100	0	100
		C2	0	100	100

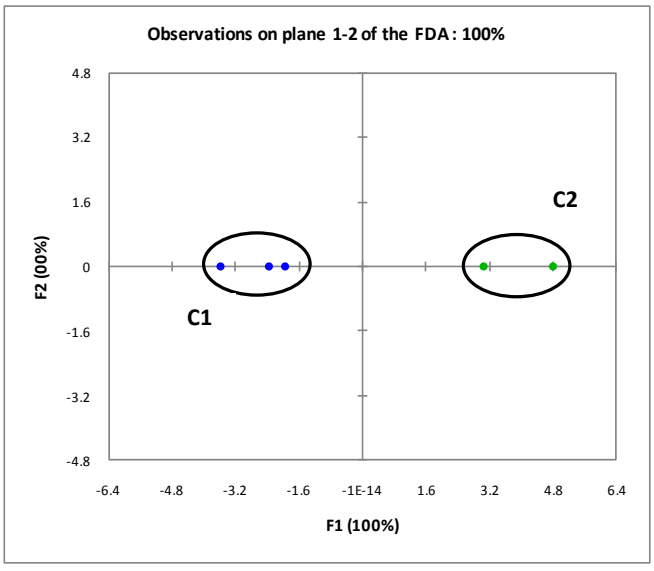
Table 5. Gap between the 2 identified classes measured from the Mahalanobis' distances.

	C1	C2
C1	0	42.957
C2	42.957	0

Table-6. Significance of the Mahalanobis' distance between the classes C1 and C2 according to Hotelling T^2 test.

Hotelling's T-Squared	F	df1	df2	p-value
107.599	107.599	1	4	0.000

Figure-3. Scatter plot showing the 2 classes constituted from the 5 upper amazon cocoa parents from the FDA.



4. Discussion

The predicting of the embryogenic performances of 5 upper amazon cocoa parents was analysed using the discriminant model of Wilks' Lambda. These 5 parents were already structured using multivariate approach [12]; nevertheless in combination with their 6 hybrids. Robinson, *et al.* [14], proposed one equation including 47 metabolites as predictors of the mature somatic embryos production in *Pinus taeda*. In oil palm, Konan, *et al.* [6] stated the modeling of the prediction of the quantity of culture biomass to be transferred and that of the number of harvested shoots at the end of each culture cycle. In Issali, *et al.* [12], 2 predicting equations predicted the membership classes of analysed observations and even those from new observations to classify. Here, only one equation namely Z1 = - 3.310 + 4.532*Ncalem allowed not only the complete discriminating of classes, but also the predicting of membership class of new observations.

As far as the PCA is concerned, for the relevance of used variables, the number of embryogenic explants (Ncalem), the embryos number per embryogenic explant (Nemb), the average embryos number per embryogenic explant (Mece) and the embryogenic percentage (Pe) were revealed to be relevant (Figure 2; Table 1). They expressed correct communality on the principal plane represented by principal components 1 and 2 (Figure 1; Table 1). They are less correlated among them. The number of callogenic explants was the only variable dropped from the study. It hindered the calculation of the determinant of the correlation matrix, and thus the outputting of tables both bearing the KMO and the result of Bartlett's test of sphericity. In contrast, in Issali, *et al.* [12], the 5 introduced variables in the analysis were all used in its rest. It may be believed that the difference of variables behaviour would be due to modification of their distributions. Indeed, the dropping here of the modalities associated with 6 hybrids, may have induced fundamental modifications of the structure of their distributions.

However, with respect to the FDA, only the number of embryogenic explants was revealed to be the most discriminant, thus relevant. Consequently, the relevantness depends on the analysis method. On the contrary, in Issali, *et al.* [12], the number of callogenic explants and the average embryos number per embryogenic explant were revealed to be relevant. Therefore, in upper amazon cocoa parents, the number of embryogenic explants will be used as a statistical predictor for the yielding of somatic embryos.

Class C1 consisting of upper amazon cocoa parents IMC67, Pa121 and Pa150 expressed the greatest callogenic potential (Figures 1, 2 and 3). Using the univariate approach, only IMC67 and Pa121 belonged to the same cluster of callogenesis [9]. Therefore, the 3 aforesaid clones could be used to make chocolate aroma, cocoa butter and theobromin from the cell callus suspensions in bioreactors.

Class C2 represented by upper amazon cocoa parents P19A and Pa13 recorded, among others, the highest number of embryogenic explants. In Issali, *et al.* [9], these 2 genotypes were characterised like highly embryogenic. Minyaka, *et al.* [15] showed that IMC67 was more embryogenic than genotypes P7, SCA6, UPA409 and IFCS. Here parent IMC67 is part of the least embryogenic class. The most likely explanation is to accept that the tissue culture was not made at the same season. Indeed, the season, the climatic period and the phenological phases influence the somatic embryogenesis expression in cocoa tree [8, 16, 17]. Therefore, parents P19A and Pa13 will be used to yield somatic embryos with a view to increase the production of beans in the fields after on-station and on-farm experiments.

The equation predicting the yielding of som atic embryos is linear function which is spelt Z1 = - 3.310 + 4.532*Ncalem. It showed that the number of embryogenic explants is the only statistical predictor reliable for the somatic embryos yielding. Likewise, Konan, *et al.* [6], in oil palm, were also evidenced linear functions predicting the quantity of culture biomass to be transferred and that of the number of harvested shoots at the end of each culture cycle. In contrast, the model proposed in Robinson, *et al.* [14] took into account 47 predictors using a Bayesian approach. Here, our predictive model incorporated only one predictor. Esse, *et al.* [18] proposed mathematical model of enzymes involved in somatic embryogenesis process. This model predicts root growth of seedlings and that of their hypocotyls. Our model only predicts embryogenic explants production, and thus somatic embryos. In Issali, *et al.* [12], the average embryos number per embryogenic explant and the number of callogenic explants were the statistical predictors more reliable. Consequently, the reliableness varied from study to study. For the prediction purposes, the observations belonging to class C1, having expressed low potential of the somatic embryos yielding, will range from 0.000 to 0.392. Their discriminating score Z1 will vary from -3.31 to -1.534 (Figure 3). Regarding the class C2, its value from the number of embryogenic explants varied from 1.3725 to 1.7402. Its discriminating score Z1 oscillated from 2.910 to 4.576 (Figure 3). Thus, all of observations equal or between these 2 scores will express high embryogenic potential. This will allow the predicting of membership class from its values of the embryogenic explants.

5. Conclusion

We postulated the hypothesis that one variable describing the somatic embryogenesis might predict accurately the embryogenic performances among the 4 embryogenesis variables out of 5 used in cocoa parents analysed here. Actually, the number of embryogenic explants was identified to be the best predictor for somatic-embryos yielding. The 2 identified classes C1 and C2 represented 2 distinct entities of morphological differentiation. The pertinentness of variables using the PCA differed from the one revealed via the FDA. Thus, it varied from analysis method to another analysis method. Cocoa parents IMC67, Pa121 and Pa150 could be used to make chocolate aroma, cocoa butter and theobromin from the cell callus suspensions in bioreactors. On the whole of 5 used variables, only the number of embryogenic explants allowed the complete discriminating of classes. Cocoa parents P19A and Pa13

must be used for embryogenesis purposes. The equation predicting the embryogenic performances is written Z1 = -3.310 + 4.532*Ncalem. It allowed the complete discriminating of 2 classes identified. The values of the best cocoa genotypes in relation to somatic embryogenesis will vary from 1.3725 to 1.7402. Their discriminating score Z1 will stretch out from 2.910 to 4.576. This equation will allow the predicting of membership set of a new observation from its values of the number of embryogenic explants.

6. Acknowledgments

We are thankful to Biscuit Cake Chocolate and Sugar Confectionery Association. The support staff of the Central Biotechnology Laboratory, namely Emmanuel Zran, Emmanuel Allé Ohoueu, Claude Kouakou Assovi and Touré Mah, are acknowledged.

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